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ULTRAHISTOPATHOLOGY OF ENCHYTRAEID OLIGOCHAETES (ANNELIDA) AFTER EXPOSURE TO PESTICIDES—A MEANS OF IDENTIFICATION OF SUBLETHAL EFFECTS?

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Abstract—1. The effects of the commonly used insecticides E605f (parathion), Ripcord 10 (cypermethrin) and the fungicide DuPont Benomyl on chloragocytes of an enchytraeid species were studied by transmission electron microscopy.

2. Animals were exposed to concentrations varying between 0.1 × and 100 × the minimum concentration recommended for agricultural application.

3. Ultrastructural changes regarding nuclei, mitochondria and other cytoplasmic compartments were obvious and were correlated to concentration and exposition time of the pesticides.

4. The importance of ultrahistopathology for terrestrial bioassays and monitoring is discussed.

INTRODUCTION

There is a considerable increase in the industrial production of organic pesticides, many of which play an important role as environmental pollutants. For example, in 1985 10,000 t. of chlorinated hydrocarbons, 13,500 t. carbamates and 24,300 t. of organophosphorous compounds were produced alone in the Federal Republic of Germany (Braunbeck and Storch, 1989). In temperate regions enchytraeid oligochaetes are highly abundant non-target species which decompose and consume decaying organic material in various types of acid soils (O'Connor, 1967; Abrahamsen, 1973; Peterson and Luxton, 1982). Cultivation of several species under laboratory conditions proved by be easy and showed enchytraeids to be well-suited ecotoxicological test organisms (Römbke, 1989), especially useful for studies on sublethal effects (Westheide *et al.*, 1989; this volume).

In connection with these investigations there was considerable interest in studying the changes caused by certain pesticides in the ultrastructure of cells and tissues. Effects of organic chemicals and heavy metals have been shown to be visible already at sublethal dosages in various organisms including oligochaetes (Fischer and Horváth, 1976; Ireland and Richards, 1977; Hagens and Westheide, 1987). Early and distinct effects are to be expected to appear first in tissues and organs involved in uptake, metabolism and detoxification of xenobiotics. In oligochaetes, cells of the epidermis, the gut and the chloragog are assumed to show early signs of toxification. In the present study structural changes were observed also in cells of the nephridia and the ventral nerve cord. Special emphasis was given to the study of chloragocytes, whose metabolic function resembles that of the vertebrate liver (Jamieson, 1981). The importance of ultrastructural changes of chloragocytes as a sensitive tool for the identification of sublethal effects of pesticides is discussed.

MATERIALS AND METHODS

The test species was a comparatively small (3 mm long) *Enchytraeus* species, the exact taxonomy of which is under investigation (*E. minutus* in Hagens and Westheide, 1987). Animals used for experiments were obtained from genetically monomorphic inbred cultures. They were maintained in Petri dishes half-filled with 1.5% nutrient agar (1 g $Ca(NO_3)_2$, 0.25 g $MgSO_4 \times 7\ H_2O$, 0.25 g KNO_3, 0.25 g KH_2PO_4, traces of $FeSO_4$ in 1-l. deionised water, pH 5; 21°C in the dark) and fed equal quantities of flaked oats. Individuals investigated were of the same age and size (7 days old: 19–21 segments and 9 days old: 29–31 segments). They were taken from bioassay experiments conducted at the same time (Westheide *et al.*, 1989). Test organisms were exposed to agar media containing the pesticide to be tested: the commercially obtained insecticides E605f (active substance 50% parathion), Ripcord 10 (active substance 10% cypermethrin) and the fungicide DuPont Benomyl (50% of the active compound). Concentrations varied between 0.1 × and 100 × the minimum recommended concentration for application converted to the surface of the Petri dish (19.6 cm^2, agar content 5 ml), i.e. 1.0 × = 0.008 μl E605f/ml medium = 4 ppm parathion; 0.012 μl Ripcord 10/ml medium = 1.2 ppm cypermethrin; 0.0118 μl DuPont Benomyl/ml medium = 5.9 ppm benomyl. Time of exposure was 2 hr, 10 hr, 2 days, 4 days, 6 days, and 10 days. Control specimens were obtained from untreated cultures.

For transmission electron microscopy an osmium ferricyanide fixation modified from McDonald (1984) was used. Whole animals were fixed in a buffered (0.05 M sodium cacodylate) solution of 2.5% glutaraldehyde for 2 hr at 21°C. After rinsing in buffer (7 changes, 1.5 hr), specimens were incubated in 0.8% potassium fericyanide in buffer for 0.5 hr at room temperature, postfixed in 0.5% OsO_4 plus 0.8% K_3 [$Fe(CN)_6$] in buffer at 4°C, rinsed once in buffer, dehydrated in a graded ethanol series and embedded in an Epon–Araldite mixture.

Ultrathin sections (Reichert Ultracut E microtome) were exclusively taken from postclitellar segments 14, 20, and 28 (3 specimens on the average). They were picked up on pioloform-coated slotgrids, stained in an LKB Ultrostainer with uranyl acetate and lead citrate, and examined in a Zeiss EM 109 transmission electron microscope. Morphometric data were obtained with a Zeiss Morphomat 40.

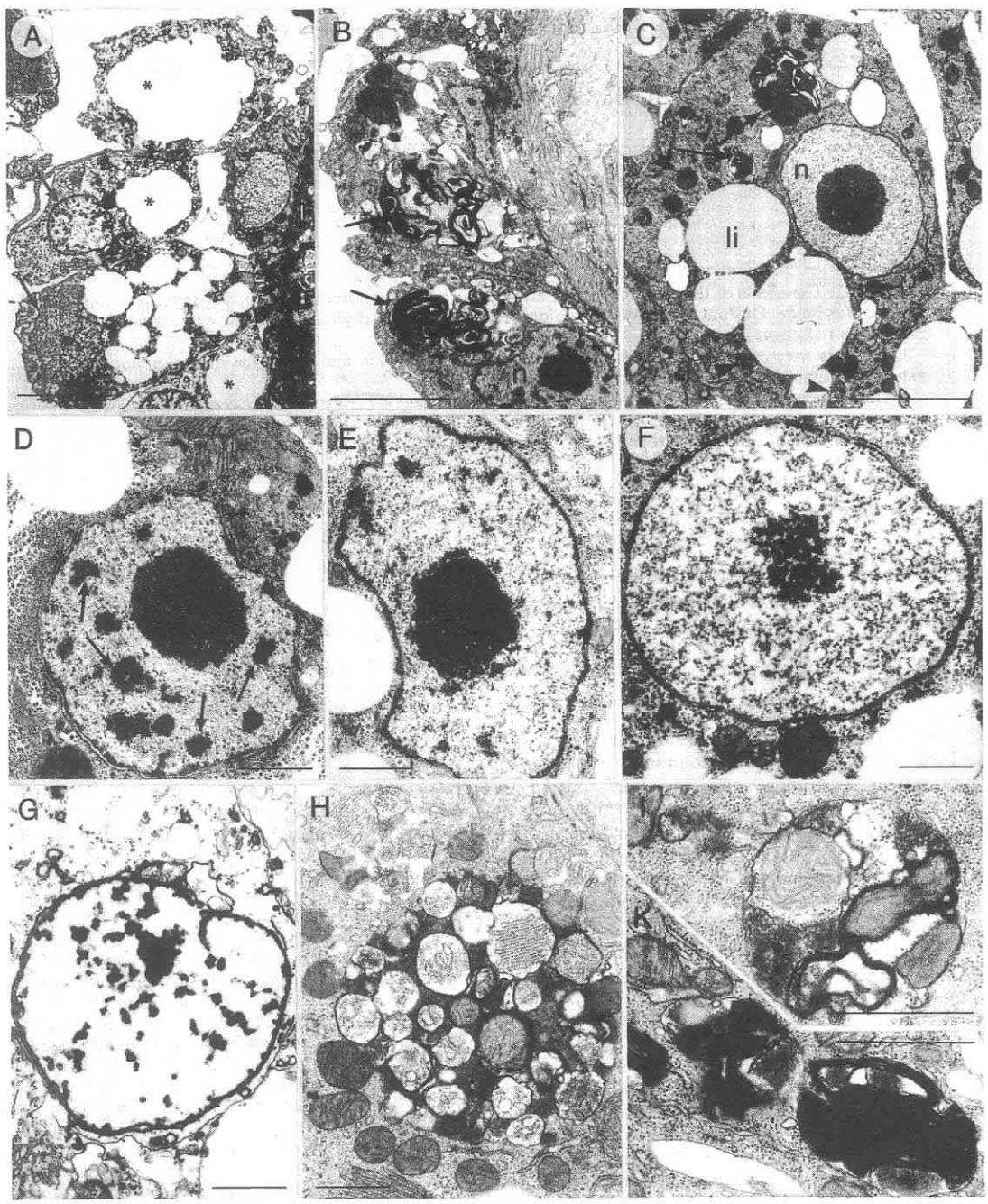

Fig. 1A–K. *Enchytraeus* sp. Chloragocytes after exposure to pesticides. A: Exposure to Ripcord 10 (0.12 μl/ml, 4 hr), chloragocytes detached from intestine (i), structural changes of nuclei (arrows) and mitochondria, fusion of lipid droplets (asterisks). B: 2 hr of exposure to 0.08 μl/ml E605f, occurrence of lamelloid bodies (arrows), nucleus (n) appears unaffected. C: 2 hr of exposure to 0.4 μl/ml E605f, autolysosomes (arrows), damaged mitochondria (arrowheads), nucleus (n) without heterochromatin; li lipid droplet. D–G: Exposure to various concentrations of Ripcord 10, nuclei; D: Control, heterochromatin distinct (arrows); E: 0.12 μl/ml for 2 hr, heterochromatin reduced; F: 0.12 μl/ml for 4 hr, dispersion of nucleolus, no heterochromatin; G: 1.2 μl/ml for 2 hr, dissolution and coagulation of karyoplasm. H–K: Exposure to E605f, autophagosomes containing mitochondria and RER and residual (lamellar) bodies. H: 0.8 μl/ml for 2 days; I, K: Application of 0.4 μl/ml for 2 hr. Scale bars in A–C equal 5 μm, in D–K 1 μm.

RESULTS

Specific differences found in chloragocytes after exposure to pesticides were numerous and diverse. The following pathological features were observed: (1) enlargement of nuclei, reduction of heterochromatin, dispersion of nucleoli (Fig. 1A–G); (2) enlargement as well as shrinkage of mitochondria, changes of mitochondrial matrices, swelling and disintegration of cristae (Fig. 1F, J); (3) multiplication and abnormal size of autophagosomes containing damaged mitochondria, RER stacks, and various different granules; (4) autolysosomes and numerous residual bodies with lamellar and myeloid contents as well as lamellar bodies of probably different origin (Fig. 1B, C, H–K); (5) increased frequency of chloragosome-like granules; (6) reduced RER; (7) unusually large lipid droplets in mature chloragocytes, some of which abnormally release their contents into the coelom (Fig. 1A); and (8) unusual numbers and dimensions of cells and their compartments.

Quantitative effects on cells and organelles, as well as the extent of these structural impairments, were directly correlated with the concentration of the pesticide tested (Fig. 1D–G). Pathological changes in chloragocytes are obvious even at sublethal concentrations; e.g. 10 hr of exposure to 0.008 μl/ml E605f or 2 hr at 0.0012 μl/ml Ripcord 10 resulted in distinctly altered mitochondria and the development of large autophagosomes containing RER, mitochondria, etc. Dimensions of single chloragocytes decreased gradually on randomly selected sections from $175 \pm 48\ \mu m^2$ (control) to $51 \pm 12\ \mu m^2$ (0.8 μl/ml E605f, 2 days of exposure). After exposure to benomyl the lipid content of chloragocytes was reduced from 74.6 ± 6.5 vol % (control) to 47.2 ± 0.7 vol % (236 μl/ml). Affected cells also had smaller mitochondria. Generally, the impact of pesticides resulted in fewer chloragocytes. However, these quantitative changes are not necessarily significant ($P \leqslant 0.01$, t-test) between successive concentration steps.

Generally, cytological changes were not substance-specific and were similar after exposure to each of the three pesticides tested. However, separation of the cuticle and breakdown of the microvillar brush border in the intestine was only observed after contact to Ripcord 10. After longer times of exposure (4, 6 or 10 days) to sublethal concentrations of pesticides, the extent of observable ultrahistopathological effects became less obvious.

DISCUSSION

Chloragocytes in enchytraeids are important in lipid synthesis, storage of metabolites and detoxification (Jamieson, 1981). They show a natural dynamic which depends on age and nutrition. This necessitated an extensive investigation of their genesis, structure, maturation dynamics and their changes to starvation and food impact. Chloragocytes pass through three distinctly distinguishable developmental stages: (1) the undifferentiated cell of the splanchnic peritoneum, (2) the ergastoplasmic cell, (3) the mature chloragocyte (Hagens, in preparation).

Very likely, uptake of pesticides in the enchytraeids cultured in agar occurs primarily through the epithelium of the gut. From here substances are easily transferred to the chloragocytes. The present study demonstrates that the ultrastructure of chloragocytes of *Enchytraeus* sp. is markedly affected by benomyl, cypermethrin and parathion. These changes are considered to be pathological and start to occur at sublethal acting concentrations. The ultrastructural changes observed in the *Enchytraeus* species are largely identical to those described for tubificid oligochaetes and earthworms (Gersch and Ude, 1971; Fischer, 1976; Fischer and Horváth, 1976; Fischer *et al.*, 1982). Also in eel hepatocytes, which functionally correspond to chloragocytes similar ultrahistopathological effects have been observed after chemical spills in the river Rhine (Braunbeck and Storch, 1989). However, quantitative differences in the pesticides' effects on the ultrastructural picture of the chloragocytes are to be expected regarding species-specificity, nutritional stage, age, and cultural conditions. Such quantitative effects are commonly observed in bioassays and other studies (Gilman and Vardanis, 1974; Segner and Storch, 1985; Westheide *et al.*, 1989).

The formation of lamellar bodies characteristic of affected cells may indicate steps within the pathway of detoxification of xenobiotics and elimination of damaged organelles. Jonsson *et al.* (1981) suggested that detoxification is ascribable to binding toxicants in lamellar bodies. These structures may arise from the RER, which is significantly reduced after exposure to pesticides (Nishizumi, 1970). The simultaneous occurrence of lamellar bodies and disappearance of RER, however, can also be explained by the autophagosomous uptake of RER and its transformation into residual bodies. This may be combined with serious disturbances of protein synthesis, as suggested by Braunbeck and Storch (1989).

The unexpected decrease of pathological effects after longer times of exposure may lead to the following hypothesis: survival of an initial phase of toxification results in an increased capability of detoxification. Damaged cells may renew organelles or the cells may be completely regenerated. However, this process of repair and regeneration requires energy, and thus reproductive capacity and life-span are significantly lower in these individuals (Bethke-Beilfuß and Westheide, 1987; Westheide *et al.*, 1989). On the other hand, the occurrence of distinct changes after short periods of exposure shows that ultrahistopathological investigations are excellent and sensitive tools for detecting early and sublethal effects caused by pesticides in animals appearing unaffected in the light microscope. The advantage of ultrahistopathological observations in comparison with bioassay studies is that effects can not only be observed under experimental and test conditions, but also in specimens collected in the field.

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